AD_________________

Award Number: W81XWH-09-1-0428

TITLE:

Exploitation of Nontraditional Crop, Yacon, in Breast Cancer Prevention Using Preclinical Rat Model

PRINCIPAL INVESTIGATOR:

Weiqin Jiang, M.D. (PI), Zongjian Zhu, M.D., Ph.D. (Co-PI)

CONTRACTING ORGANIZATION:

Colorado State University
Fort Collins, CO 80523-0001

REPORT DATE:

July, 2010

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*
01-07-2010

2. REPORT TYPE
Annual

3. DATES COVERED *(From - To)*
1 JUL 2009 - 30 JUN 2010

4. TITLE AND SUBTITLE
Exploitation of Nontraditional Crop, Yacon, in Breast Cancer Prevention Using Preclinical Rat Model

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0428

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Zhu, Zongjian and Jiang, Weiqin

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Colorado State University

Fort Collins, CO 80523-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and
Materiel Command

Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Yacon has recently been introduced into farmer's markets and natural food stores in the US, but its preventive activity for breast cancer has rarely been evaluated. Objective are to determine the effect of dietary yacon on 1-methyl-1-nitrosourea (MNU) induced mammary carcinogenesis in rat; to evaluate the circulating factors and their association with the carcinogenesis; and to determine cellular signaling pathways – HDAC and downstream targets - AMPK/Akt-mTOR and ghrelin-IGF1 axis. Mammary carcinogenesis was initiated by injection of female rats with 50 mg MNU/kg body weight (i.p.) at 21 days of age. One week later, the rats were fed diets containing yacon powder at 0%, 15%, 30% or 60% (30 rats/group) for 8 weeks, respectively. Results showed that dietary yacon reduced the promotion and progression of MNU-induced mammary carcinogenesis in rat, which is associated with downregulation of IGF-1/HDAC/Akt/mTOR signaling pathway and anti-inflammation, i.e. reduction of plasma IL-6, TNFα, and C-reactive protein. Future work will focus on the effect of yacon on obesity and anti-inflammation that are factors indused breast cancer. The study will provide crucial biological information to complement the knowledge of natural functional foods that is relevant to a number of foods rich in non-digestible, fermentable oligosaccharides.

15. SUBJECT TERMS
Yacon, mammary carcinogenesis and prevention, anti-inflammation,

16. SECURITY CLASSIFICATION OF: UNCLASSIFIED			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	18	19b. TELEPHONE NUMBER *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

	Page
Introduction……………………………………………………………………….……..	1
Body………………………………………………………………………………..	2-4
Key Research Accomplishments………………………………………….………..	5
Reportable Outcomes……………………………………………………………	6
Conclusion……………………………………………………………………………	7
References…………………………………………………………………………….	8
Appendices……………………………………………………………………………	9
Supporting Data……………………………………………………………………….	10-15

INTRUCTION

Yacon contains a large amount of non-digestible oligosaccharide called inulin that belongs to a class of carbohydrates known as fructans [1]. Inulin-type fructans (ITF) can produce butyrate and decreases the rate of aberrant crypt foci (ACF), a pre-neoplastic lesion found in colon [2]. Butyrate modulates gene transcription by inhibiting histone deacetylases (HDAC) [2]. Cancer cells appear to be more sensitive than non-transformed cells to HDAC inhibitory compounds. In addition, the yacon ITF promotes satiety and retard the absorption of food-derived energy via reducing a gastrointestinal peptide-***ghrelin*** that is a growth hormone secretagogue [3;4]. Reduction of serum ghrelin results in decreases of IGF-1 [5]. Subsequently, the PI3K/Akt-mTOR signaling pathway will be inactivated via increasing AMPK activity and its downstream events, inhibition of cell proliferation and induction of cell apoptosis will be observed. However, few studies have been shown the effect of the ITF on breast cancer. ***The hypotheses*** were that treatment with yacon will: 1) dose-dependently reduce the incidence and multiplicity of chemically-induced pre-malignant and malignant mammary tumors in rats, 2) increase butyrate and decrease ghrelin and insulin-like growth factor 1 (IGF-1) in the blood, 3) inhibit HDAC, and downregulate Akt-mammalian target of rapamycin (mTOR) signaling pathway through upregulated AMP activated kinase (AMPK) – a energy metabolic sensor, eventually decreasing cell proliperation and increasing apoptosis. **Objective were:** 1) To determine ***mammary carcinogenic responses to yacon*** in experimental animals. 2) To evaluate the association of the ***circulating mediator, gastrointestinal peptide, and growth factors*** with the carcinogenic responses in rats fed dietary **yacon**. 3) To determine the ***HDAC*** and identify AMPK/Akt-mTOR ***cell signaling pathway*** that were downstream targets of HDAC and ghrelin-IGF1 axis. Mammary carcinogenesis was initiated by injection of female rats with 50 mg of 1-methyl-1-nitrosourea/kg body weight (i.p.) at 21 days of age. One week later, the rats were fed diets containing yacon powder at 0%, 15%, 30% or 60% (30 rats/group) for 8 weeks, respectively. Rats were palpated for mammary tumors and effects of **yacon** on the incidence, multiplicity and latency of mammary carcinomas were evaluated. Plasma was collected to determine blood glucose, insulin, leptin, butyrate, ghrelin, and IGF-1 as well as inflammatory factors. The tumor and liver tissues were used for histological evaluation and molecular biological assessments including HDAC and regulators that were associated with AMPK/Akt-mTOR pathway in mammary gland pathologies. Differences between diet groups were statistically analyzed by ANOVA, Chi-Square, survival and correlation analyses. **Yacon** can be readily introduced into the food supply for some specific cancer prevention and retard recuration since it has been in the market. The study will provide crucial biological information to complement the knowledge of natural functional foods that is relevant to a number of foods rich in non-digestible, fermentable oligosaccharides. Consequently, the new type of functional food will significantly contribute to the goal of proventing initiation of breast cancer and reduce survivor recuration because it is natural, tolerantable and acceptable for the clinic patients and general population in aditional to benefit to obesity, diabetes II and cadiovascular diseases.

BODY

Task 1. To determine mammary carcinogenic responses to yacon in experimental animals.

a. Initiate Pre-clinical model for breast cancer in rat induced by 1-methyl-1-nitrosourea (MNU) and feed yacon in diet (Month 1-3).

One hundred twenty female Sprague Dawley rats were obtained at 20 days of age. They were injected with MNU (50 mg/kg body weight) at 21 days of age as previously described by us[6]. Following carcinogen administration, rats were randomized to one of four diet groups and 30 rats per group: AIN 93G (control) or AIN 93G supplemented with 150 g, 300 g or 600 g of yacon root powder/kg diet. Inulin concentration in the yacon is 4.66 g/100 g, which was analyzed by Warren Analytical Laboratory (Greeley, CO). The dose of 15% - 60% dietary yacon provided 0.7% - 2.8% of inulin-type fructans in diet) is about 0.5 – 2.0 g inulin-type fructans/kg body weight rat, which is the acceptable dose for human. Experimental diets were fed to the rats beginning one week post carcinogen administration in order to determine the inhibitory effect of yacon on promotion and progression of mammary carcinogenesis. All of rats were housed three per cage and weighed 1-2 times per week. At the end of study, no significant difference was observed in the final body among 4 dietary groups (ANOVA, P=0.165, see figure and table 1 in supporting data section). The average of body weight is 225, 222, 217 or 215 gram for groups of control, 15%, 30% or 60% yacon. From three weeks of post carcinogen until study termination, rats were palpated twice per week for detecting mammary tumors. A log was kept of all detected tumors and their locations. Experiment was terminated at 9 week of post carcinogen. At necropsy, the log was used to record all grossly visible tumors and to assist with confirmation of all palpable tumors. All tumors detected at necropsy were excised and weighed. One portion of excised tumors was processed for histological classification and the remainder part of each tumor was snap-frozen in liquid nitrogen and were available for the proposed mechanistic studies in Task 3. Abdominal-inguinal mammary glands from both sides of animal were excised. One side were spread out on a glass and fixed in 10% formalin and another side were spread out on a film, which was sealed in a bag and snap-frozen in liquid nitrogen for molecular analysis (in Aim 3). Liver was also excised and snap-frozen in liquid nitrogen. At the end of this experimental period, the samples for all of the Tasks were collected and stored at -80ºC for further analyses.

b. Process mammary glands and mammary tumors for the histological and immunohistological evaluations.

All mammary glands whole mounts were stained for further analysis. All tumors excised at necropsy were processed for histological classification. Briefly, all lesions were put in cassettes individually. The cassettes were processed through toluene and molten paraffin. Then, the lesions were embedded in paraffin and cut at 4 um sections that were placed onto glass microscope slides. Sections were heat immobilized and then stained using an H&E protocol. At the end of this experimental period, all of slides were stained and ready for further evaluation.

c. Evaluate the mammary lesions histologically and summarize the mammary carcinogenic responses to dietary yacon.

All of the tumors were stained by H&E and were histologically diagnosed under microscope. The tumors that were detectable by palpation and that were histologically confirmed to be mammary adenocarcinoma (AC) were summarized to produce the incidence, multiplicity, weight and latency curves. At the end of this period, we found that palpated cancer incidence was significantly reduced by dietary yacon (Chi-square test, p=0.05) while multiplicity (palpated cancer number/rat) was not significantly different among 4 groups (Kruskal-Walls Test, p=0.247). The cancer weight was significantly lower in 30% yacon group (Kruskal-Walls Test, compared to control, p=0.035; compared to 15% yacon group, p=0.033). Latency of palpated carcinomas was delayed by dietary yacon (p=0.07, Mantel test, see figure 2 and table 1 in supporting data section).

Task 2. To evaluate the association of circulating mediator, gastrointestinal peptide, and growth factors with the carcinogenic responses in rats fed dietary yacon (month 7-9).

Blood samples were collected at necropsy for the evaluation of circulating butyrate, ghrelin, IGF-1, glucose, insulin, leptin, and inflammatory factors from the study described in Task 1.

a. Determine the circulating mediator – butyrate.

Butyrate will be determined by gas chromatography and the assays are on the way.

b. Determine plasma gastrointestinal pepide – ghrelin.

Plasma active ghrelin was determined and no significant difference was observed among 4 groups (P=0.753, ANOVA) (see figure 3 and table 2 in supporting data section).

c. Determine serum growth factors – Glucose, insulin, leptin, IGF-1, and inflammatory fators - C-reactive protein, IL-6 and TNFα.

Plasma glucose, insulin, leptin, and insulin-like growth factor 1 (IGF-1) and inflammatory factors were determined. Compared to control and 15% yacon groups, plasma glucose in 60% yacon group was significantly higher ($p<0.03$, Bonferroni Test). No significant difference was observed in plasma insulin among 4 groups (P=0.755, ANOVA). Plasma leptin was lower in 30% yacon group. But no significant difference was observed in plasma leptin among 4 groups (P=0.097, ANOVA). Plasma IGF-1 were significantly different (ANOVA, p=0.05) among 4 groups. Regression analysis p=0.005. Compared to the control group, the plasma level of IGF-1 was significantly decreased in 60% yacon group (P=0.05, Bonferroni Test). In addition, plasma C-reactive protein (CRP) and IL-6 were significantly decreased by dietary yacon ($P<0.0001$, ANOVA). Compared to control group, plasma CRP and IL-6 in 30% and 60% yacon groups were significantly reduced ($p<0.001$, Bonferroni Test). Compared to 15% yacon group, CRP in 60% yacon group was significantly reduced ($p<0.001$, Bonferroni Test). Plasma TNFα was also significantly decreased by dietary yacon (P=0.01, ANOVA). Compared to control group, plasma TNFα in 30% and 60% yacon groups was significantly reduced ($p<0.05$, Bonferroni Test). (see figure 3 and table 2 in supporting data section)

d. Summarize the data and identify the association of all circulating factors with the carcinogenic response to dietary yacon.

At the end of this period, the effect of dietary yacon on the levels of circulating glucose, insulin, leptin, ghrelin, IGF-1 and inflammatory factors were ascertained. And the association between circulating factors (glucose, insulin, leptin, ghrelin, and IGF-1) and the carcinogenic responses (the incidence and multiplicity of adenomas) were identified. As expected, the incidence, multiplicity and weight of mammary carcinomas were significantly positive-correlated to each other. These carcinogenic responses were also positive-correlated to plasma IL-6. And, the carcinogenic responses were significantly negative-correlated to the latency of the carcinomas as well as to plasma glucose, IL-6 and TNFα ($p<0.001$) while no significant correlation was observed between carcinogenic responses and other circulating factors. However, significant correlations were observed among the circulating factors, which were positive correlations between insulin and IGF-1, leptin, or glucose as well as between C-reactive protein and IGF-1 or leptin ($p<0.01$), also between IL-6 and C-reactive protein or IGF-1; and negative correlations between leptin and active ghrelin ($p<0.01$). (see table 3 in supporting data section)

Task 3. To determine the HDAC and identify cell signaling pathway that were downstream targets of HDAC and ghrelin-IGF1 axis.

a. Determine the protein levels of HDAC, PI3K, Akt, mTOR and other related regulators in PI3K/Akt-mTOR pathway.

Frozen mammary gland, mammary tumors and liver of control and 30% yacon groups collected under Task 1 were homogenized for determining the levels of HDAC, Akt, mTOR, AMPK by western blotting. It has been observed that HDAC in all of three tissues were decreased in 30% yacon group compared the control group. Significant reduction was observed in both mammary and mammary carcinomas ($p<0.05$). There was no significant difference in Akt and AMPK of three

tissues between control and yacon group. However, phosphorylated mTOR and mTOR were significantly decreased in mammary carcinomas of 30% yacon group to compared with the control group ($p<0.0001$) while only mTOR was significantly reduced in mammary gland of 30% yacon group. (see figure 4 and table 4 in supporting data section)

b. Summarize the data and identify the regulations of HDAC and Akt-mTOR/AMPK pathway by HADC and ghrelin-IGF1 axis.

The current data suggest that dietary yacon inhibits HDAC and downregulates mTOR signaling pathway in mammary gland and mammary carcinomas. The changes were more obvious in the mammary tumors than in the mammary gland. In addition, the reduction of plasma IL-6, TNFα and C-reactive protein by dietary yacon suggests that yacon has impact on inflammation. Further investigation will focus on yacon anti-inflammation effect and its association with the inhibition of breast cancer in high inflammatory status, such as metabolic syndrome in women and in pre-clinical animal model.

Taken together, our data indiate that yacon shows inhibitory effect on the promotion and progression of chemically-induced mammary carcinogenesis in rat. The inhibition is assicoated with downregulation of IGF-1/HDAC/Akt/mTOR signaling pathway and with anti-inflammation.

KEY RESEARCH ACCOMPLISHMENTS

- Dietary yacon reduced the promotion and progression of mammary carcinogenesis in rat.
- The inhibition is assicoated with downregulation of IGF-1/HDAC/Akt/mTOR signaling pathway, and with anti-inflammation

.

REPORTABLE OUTCOMES

- Presentation: The current results will be presentated at 9th Annual AACR International Conference on Frontiers in Cancer Prevention Research (November 7-10, 2010, Pennsylvania Convention Center, Philadelpha, PA).
- Paper: A manusscript titled "Effect of Dietary Yacon on Promotion and Progression of Chemically-Induced Mammary Carcinogenesis in Rat" will be submitted to Cancer Prevention Resaerch.

CONCLUSION

Results showed that dietary yacon potentially reduced the promotion and progression of chemically-induced mammary carcinogenesis in rat, which is associated with downregulation of IGF-1/HDAC/Akt/mTOR signaling pathway and with anti-inflammation. Future work will focus on the effect of yacon on obesity and anti-inflammation, which are key risk factors for breast cancer.

REFERENCES

1. Roberfroid,M.B. (2005) Introducing inulin-type fructans. *Br.J.Nutr.*, **93 Suppl 1**, S13-S25.

2. Pool-Zobel,B., van,L.J., Rowland,I., and Roberfroid,M.B. (2002) Experimental evidences on the potential of prebiotic fructans to reduce the risk of colon cancer. *Br.J.Nutr.*, **87 Suppl 2**, S273-S281.

3. Gale,S.M., Castracane,V.D., and Mantzoros,C.S. (2004) Energy homeostasis, obesity and eating disorders: recent advances in endocrinology. *J.Nutr.*, **134**, 295-298.

4. Geyer,M., Manrique,I., Degen,L., and Beglinger,C. (2008) Effect of yacon (Smallanthus sonchifolius) on colonic transit time in healthy volunteers. *Digestion*, **78**, 30-33.

5. Kojima,M., Hosoda,H., and Kangawa,K. (2001) Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. *Horm.Res.*, **56 Suppl 1**, 93-97.
6. Zhu, Z., Haegele, AD, Thompson, HJ. (1997) Effect of caloric restriction on pre-malignant and malignant stages of mammary carcinogenesis. Carcinogenesis, 18: 1007-1012.

APPENDICES: N/A

SUPPORTING DATA

Figure 1. Final body weight (BW). Group 1, control; 2, 15% yacon; 3, 30% yacon; and 4, 60% yacon. The error bars are SEM.

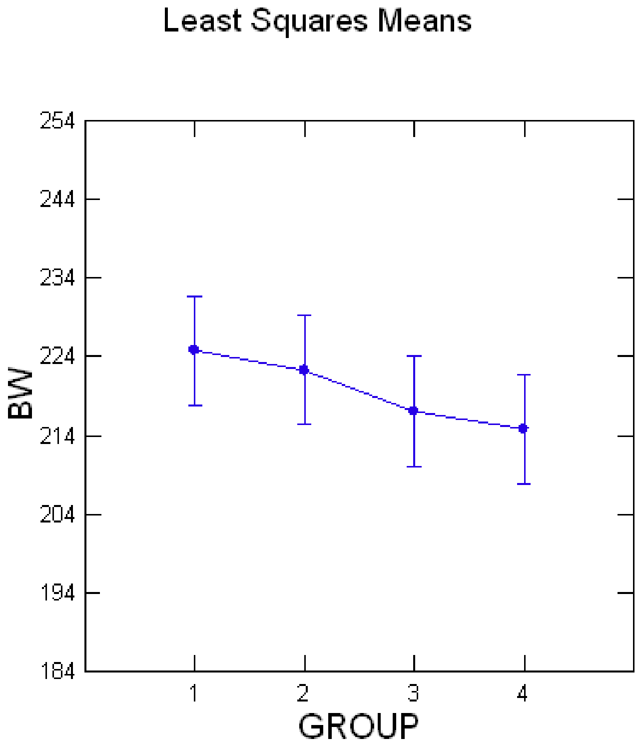


Figure 2. Latency of cancinoma incidence

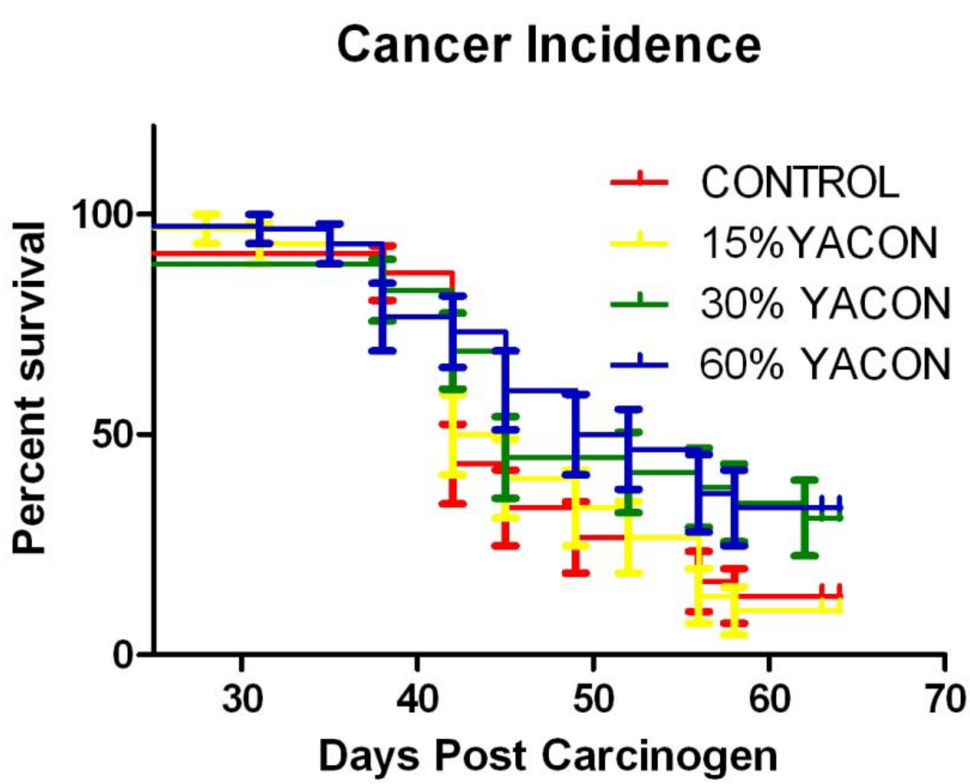
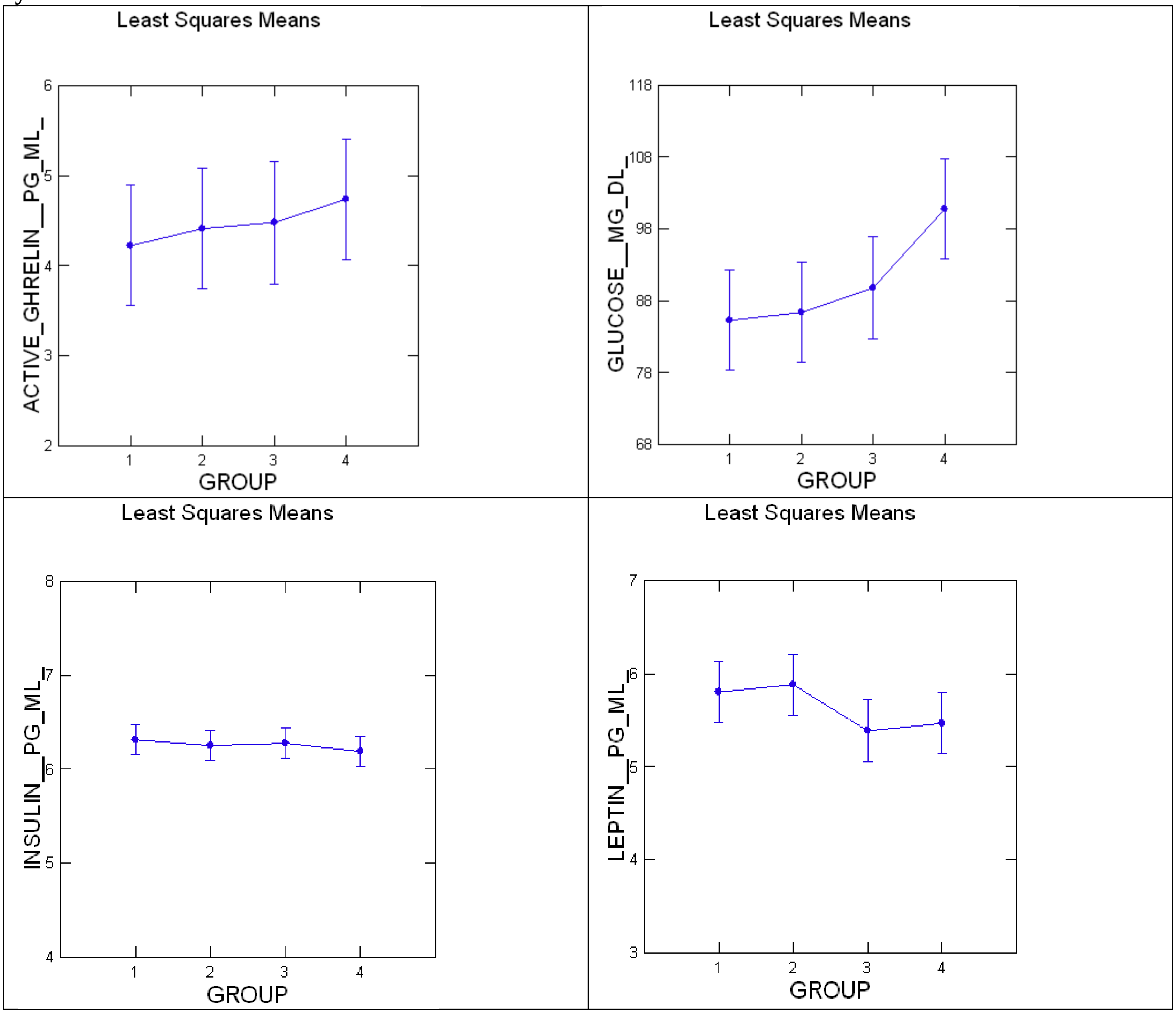


Figure 3. Circulating glucose and hormones. Group 1, control; 2, 15% yacon; 3, 30% yacon; and 4, 60% yacon. The error bars are SEM.



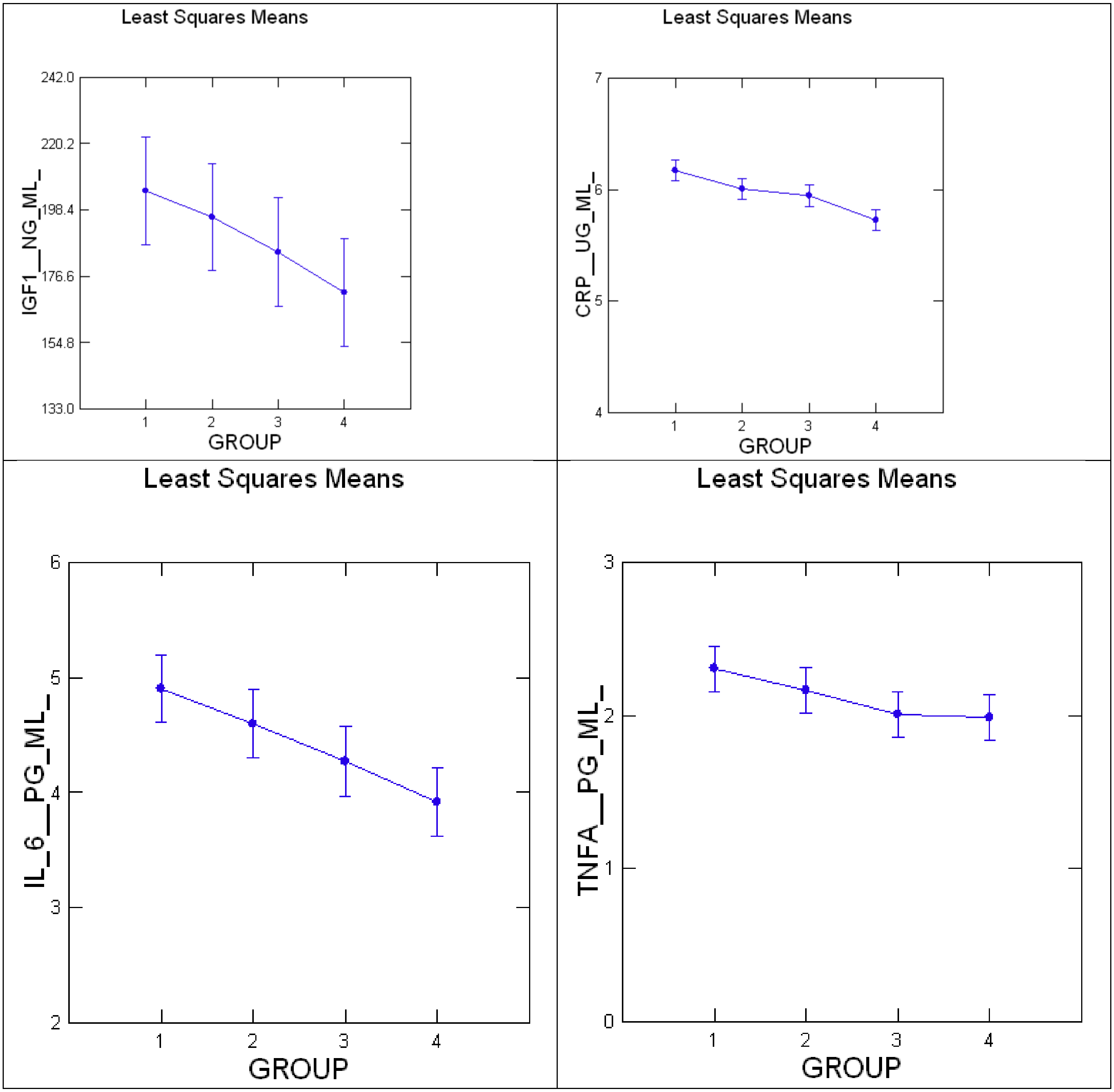

Least Squares Means
242.0
220.2
198.4
176.6
154.8
133.0
IGF1__NG_ML_
1 2 3 4
GROUP
Least Squares Means
7
6
5
4
CRP__UG_ML_
1 2 3 4
GROUP
Least Squares Means
6
5
4
3
2
IL_6__PG_ML_
1 2 3 4
GROUP
Least Squares Means
3
2
1
0
TNFA__PG_ML_
1 2 3 4
GROUP

Figure 4. Western blotting

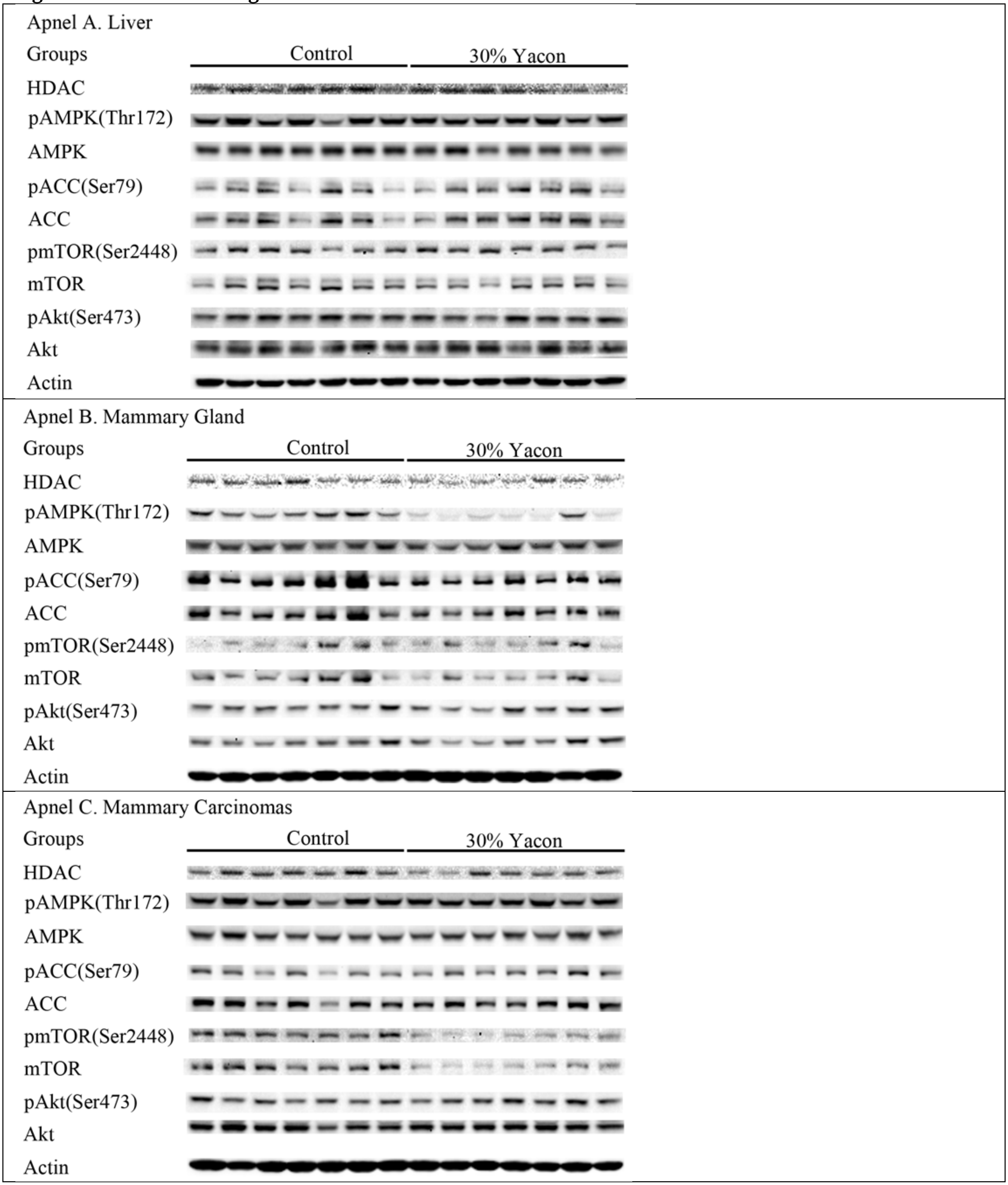


Table 1. Effect of Dietary Yacon on Final Body Weight and Mammary Carcinogenic Responses to Chemical Carcinogen[1]

	Control	15% Yacon	30% Yacon	60% Yacon	P Value
Body Weight (g)	225 ± 4	222 ± 4	217 ± 3	215 ± 3	>0.05
Cancer Incidence (%)	87%[a]	90%[a]	69%[b]	67%[b]	0.05
Cancer No./Rat	2.53 ± 0.32	2.53 ± 0.35	1.86 ± 0.34	2.27 ± 0.47	>0.05
Cancer Weight (g)	3.58 ± 0.74[a]	3.31 ± 0.65[a]	1.55 ± 0.37[b]	2.12 ± 0.641[ab]	0.009
Cancer Lantency (day)	47 ± 2	46 ± 2	51 ± 2	52 ± 2	0.07

[1]Values are mean ± SEM except cancer incidence. Significant diccerence among groups is indicated by different superscript (a or b).

Table 2. Circulating glucose and hormones[1]

	Control	15% Yacon	30% Yacon	60% Yacon	P Value
Glucose (mg/dL)	85 ± 4[a]	86 ± 4 [a]	90 ± 3 [ab]	101 ± 4 [b]	0.009
IGF-1 (ng/mL)	205 ± 9 [a]	196 ± 10 [ab]	185 ± 9 [ab]	171 ± 7 [b]	0.05
Insulin (pg/mL)	603 ± 48	598 ± 73	595 ± 60	526 ± 44	0.755
Leptin (pg/mL)	485 ± 71	524 ± 113	298 ± 49	347 ± 63	0.097
Active Ghrelin (pg/mL)	240 ± 46	247 ± 44	259 ± 49	311 ± 60	0.753
C-reactive Protein (ug/mL)	497 ± 28[a]	420 ± 19 [ab]	393 ± 18 [b]	317 ± 17 [c]	<0.0001
IL-6 (pg/mL)	185 ± 32 [a]	126 ± 16 [ab]	89 ± 10 [bc]	76 ± 13 [c]	<0.0001
TNFα (pg/mL)	9.2 ± 0.8 [ab]	13 ± 3 [a]	7.6 ± 0.3 [b]	8.2 ± 0.9 [ab]	0.01

[1]Values are mean ± SEM. Significant diccerence among groups is indicated by different superscript (a, b or c).

Table 3. Correlations among mammary carcinogenic responses and circulating factors[1]

	Cancer Incidence	Cancer No./Rat	Cancer Weight	Cancer Lantency	Glucose	IGF-1	Leptin	IL-6	TNFα	CRP
Cancer Incidence	1.00									
Cancer No./Rat	0.73*	1.000								
Cancer Weight	0.72*	0.84*	1.00							
Cancer Lantency	-0.72*	-0.74*	-0.83*	1.00						
Glucose	-0.21*	-0.10	-0.08	0.19*	1.00					
IGF-1	-0.05	-0.18	-0.20*	0.19*	0.12	1.00				
Leptin	0.07	-0.03	-0.01	-0.12	0.14	013	1.00			
IL-6	0.24*	0.34*	0.27*	-0.27*	-0.12	-0.01	0.16	1.00		
TNFα	0.15	0.12	0.28*	-0.23*	-0.15	-0.02	-0.04	0.23*	1.00	
CRP	0.003	0.04	0.05	-0.01	-0.16	0.24*	0.22*	0.14	0.04	1.00

[1]Values are correlations. *p<0.05.

Table 4. Effects of dietary yacon on cellular signaling pathways

	Liver		Mammary Gland		Mammary Carcinomas	
Groupss	Control	30% Yacon	Control	30% Yacon	Control	30% Yacon
HDAC	100	92	100	80*	100	74*
pAMPK^{Thr172}	100	113	100	39*	100	114
AMPK	100	88*	100	91	100	128
pAMPK/AMPK	100	133	100	44*	100	96
pACC^{Ser79}	100	146	100	76*	100	130
ACC	100	139	100	84	100	110
pACC/ACC	100	103	100	92*	100	135
pmTOR$^{Ser2448)}$	100	96	100	140	100	37*
mTOR	100	89	100	63	100	36*
pmTOR/mTOR	100	110	100	330*	100	118
pAkt$^{(Ser473)}$	100	101	100	101	100	130
Akt	100	102	100	94	100	112
pAkt/Akt	100	106	100	105	100	120

[1]Values are the percent of control. Compared to the control group, *p<0.05.